Immunoexpression of Ki67 and cyclin D1 in oral squamous carcinomas

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Abstract
In this study, we analyzed Ki67 and cyclin D1 immunoexpression in 44 oral squamous cell carcinomas from various anatomical sites. Ki67 immunoreaction was identified in all analyzed cases and presented an index of proliferation of 22% for well-differentiated carcinomas, 32% for moderately differentiated and 53% for the poorly differentiated ones. In case of cyclin D1, the mean positivity index was 8% for well-differentiated carcinomas, 18% for moderately differentiated and 34% for the poorly differentiated carcinomas. The analyzed biomarkers prove useful to identify lesions with poor differentiation and invasive behavior.

Keywords: oral squamous carcinoma, Ki67, cyclin D1, immunohistochemistry.

Introduction
Head and neck cancer is the sixth most common cancer [1], representing 3% of all localizations. In 48% of these cases, the tumors were located in the oral cavity and 90% are squamous cell carcinomas [2].

The pathogenesis of oral squamous cell carcinoma (OSCC) involves many different ways and therefore molecular changes underlying tumor progression are obscure and are subject of many studies. Over time many parameters related to the host or the tumor were studied unsuccessfully, in attempting to predict the disease evolution. However, in recent years, several biomarkers that provide prognostic information useful in the management of head and neck squamous carcinomas have been identified, such as p53 tumor suppressor gene mutation, proto-oncogene cyclin D1 and EGFR overexpression, which have been associated with unfavorable prognosis [3–7].

This study aimed to evaluate the expression of Ki67 and cyclin D1 in order to identify their role in oral carcinogenesis, following possible correlations between these and the analyzed morphological or clinical parameters.

Materials and Methods
The study included 44 surgical pieces of OSCC, diagnosed in the Pathology Laboratory of the Emergency County Hospital of Craiova. Tissue fragments were fixed in 10% formalin and processed by the usual histopathological technique for paraffin embedding and Hematoxylin and Eosin staining. Clinical and histological data were analyzed and the classification of lesions was done according to the latest WHO criteria [8].

The immunohistochemical processing was made on serial sections, using monoclonal antihuman antibodies (Table 1).

Table 1 – Panel of antibodies used

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Dilution</th>
<th>Antigen retrieval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin D1</td>
<td>EP12, Dako</td>
<td>1:100</td>
<td>Tris-EDTA buffer, pH 9</td>
</tr>
<tr>
<td>Ki67</td>
<td>MIB1, Dako</td>
<td>1:100</td>
<td>Citrate buffer, pH 6</td>
</tr>
</tbody>
</table>

The work system for immunohistochemical reactions was the LSAB+ System-TRP (DAKO), and the signal detection was performed with DAB (3,3’-diaminobenzidine, Dako).

Ki67 proliferation index (PI) and the positivity index (PoI) for cyclin D1 were calculated by reporting the number of positive cells (nuclei) to the total number of cells (positive and negative), the result being multiplied by 100. Were counted (using the ×40 objective) at least 500 nuclei for each case being interpreted as positive the brown to black nuclei [9]. For the immunquantification of Ki67 and cyclin D1 we used a semi-quantitative evaluation system, consisting of three levels: <10%, 10–50%, and >50% labeled cells. Negative external control staining was obtained by omitting the primary antibodies.

Statistical analysis of the results was performed with SPSS 10 software using the chi-square test for dependence assessment. The acquisition of the images was performed with a Nikon Eclipse E600 microscope and the Lucia 5 software.

Results
The study group consisted of 44 squamous cell carcinomas of the oral mucosa, diagnosed in patients aged between 40–78 years, from which more than three quarters were male patients (Table 2).
Table 2 – Clinicopathological parameters

<table>
<thead>
<tr>
<th>Clinicopathological parameters</th>
<th>No. of cases</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age [years]</td>
<td>39–50</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>50–60</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>&gt;60</td>
<td>12</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>13</td>
</tr>
<tr>
<td>Localization</td>
<td>Lip</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Tongue</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Palate</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Gingiva</td>
<td>1</td>
</tr>
<tr>
<td>Differentiation grade</td>
<td>Well-differentiated</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Moderately differentiated</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Poorly differentiated</td>
<td>15</td>
</tr>
<tr>
<td>KI67</td>
<td>Positive</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>–</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>Positive</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>20</td>
</tr>
</tbody>
</table>

The analysis of KI67 immunoreactivity indicated positivity in all investigated cases, but the percentage of immunostain index was different, correlated with the degree of neoplastic differentiation. In contrast, the cyclin D1 immunostain was present for only 24 (54.5%) of the analyzed cases.

The study of KI67 immunoreactivity for the 12 cases of well-differentiated OSCC revealed positivity in all investigated cases, on limited areas and with random distribution or to the peripheric cells of neoplastic islands. The immunoreaction corresponded to a PI-KI67 value of 22%. The examination of the immunostain for moderately differentiated OSCC showed higher values of medium PI-KI67, respectively 32%, than the well-differentiated ones. We noted that KI67 was expressed intensely in the peripheral areas of carcinoma islands (Figure 1a). The study of KI67 expression in poorly differentiated OSCC indicated a high medium PI-KI67 of 53% (Figure 1, b and c), with distribution throughout the tumor islands.

Cyclin D1 immunoreactivity was present at the nuclear level. Negative cases corresponded to well and moderately differentiated carcinomas. We observed positivity in less than 10% of tumor cells for four (16.6%) cases who belonged to well and moderately differentiated carcinomas. Immunoreaction for 10–50% and >50% tumor cells was identified in eight (33.4%) cases, and respectively in 12 (50%) cases, that belongs to moderate and poorly differentiated carcinomas. Cyclin D1 medium PoI was 8% for well-differentiated carcinomas, 18% for the moderately differentiated, and 34% for the poorly differentiated ones (Figure 1, d–f).

Statistical analysis indicated a significant correlation of KI67 and cyclin D1 immunoreactivity with tumor grade ($p<0.05$, chi-square test). We have not identified statistical associations of these biomarkers with other clinical analyzed parameters.

![Figure 1 – Oral squamous carcinoma: (a) Well-differentiated, IP – 15% (KI67 immunostaining, ×200); (b) Moderately differentiated, IP – 25% (KI67 immunostaining, ×200); (c) Poorly differentiated, IP – 40% (KI67 immunostaining, ×200); (d) Well-differentiated, IPo – 12% (cyclin D1 immunostaining, ×200); (e) Moderately differentiated, IPo – 15% (cyclin D1 immunostaining, ×200); (f) Poorly differentiated, IP – 30% (cyclin D1 immunostaining, ×200).](image-url)
Discussion

Uncontrolled proliferation of cells is one of the most important biological mechanisms associated with oncogenesis [10], a number of studies indicating that proliferation has prognostic value in a variety of tumors. Cell proliferation markers were classified into three categories: markers of growth, cell cycle phase specific markers and time markers of cell cycle [11]. Growth fraction is the ratio of cells growing in cell cycle that can be easily identified with Ki-67 or MIB-1 antibody, by identifying antigen expression in the G1, S, G2 and M phases of the cell cycle.

In this study, the Ki67 immunoexpression analysis indicated positivity in all cases, but with different percentage index (PI), which correlated with the degree of differentiation of neoplasia. Thus, the Ki67 immuno-reaction in well-differentiated OSCC revealed a medium PI-Ki67 of 22%, in moderately differentiated OSCC PI-Ki67 was 32%, and a medium value of 53% in case of poorly differentiated carcinomas.

The literature data on the relationship of Ki67 with OSCC tumor grade indicated different results. A study on cell proliferation in the OSCC reported that Ki-67 proliferation index was significantly higher in tumors with low histological grade of differentiation [7]. Other studies have communicated that histological type and clinical staging are not correlated with Ki67 value. Thus, there are studies on indices of proliferation of squamous carcinomas of the head and neck who reported that poorly differentiated tumors had low or intermediate Ki67 index (<30%), suggesting that proliferative activity in squamous carcinomas of head and neck is not always related to pathological features [12].

Extensive studies about the correlation of Ki67 expression and prognosis could not find any association between these parameters [13, 14], probably because Ki67 is a marker of total fraction of proliferating cells, corresponding both to the constantly proliferating cells and proliferating cells for terminal differentiation [14].

Type D cyclins, including cyclin D1, together with p53 protein play an essential role in controlling cell cycle by controlling the progression in G1 phase, being expressed in G1 and S phases of the cell cycle [15]. Their overexpression leads to loss of control by accelerating cell cycle G1 phase [15]. Some studies indicate that cyclin D1 is operating as a negative regulator of cell proliferation [16–18].

Cyclin D1 is overexpressed in many human cancers due to gene amplification or translocations targeting locus D1 (CCND1) of chromosome 11q13. Functional alterations of this protein may play an important role in the carcinogenesis of the head and neck squamous carcinomas and in the clinical course of tumors [19].

The analysis of cyclin D1 immunoexpression revealed positivity in 24 cases (54.5%), the over 10% tumor positivity index (PoI) being associated with moderate and poorly differentiated carcinomas.

The literature data indicates different values of positivity beyond that found by us: 32% [20], 36.7% [21], 63.8% [22], and even 70.7% [23]. Similar studies for cyclin D1 communicate positivity in 46% of OSCC and for 83% of premalignant lesions [24].

Other studies of cyclin D1 immunoexpression report faster and more frequent recurrence of the disease [6, 20, 22] and decreased survival [25–27], being an indicator of adverse prognosis, independent of other known prognostic factors [6, 28, 29].

Conclusions

The immunohistochemical study of oral squamous carcinomas indicated overexpression of cyclin D1 and Ki67 in moderately and poorly differentiated lesions. Ki67 and cyclin D1 may prove useful for selecting patients requiring intensive treatment.

References


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